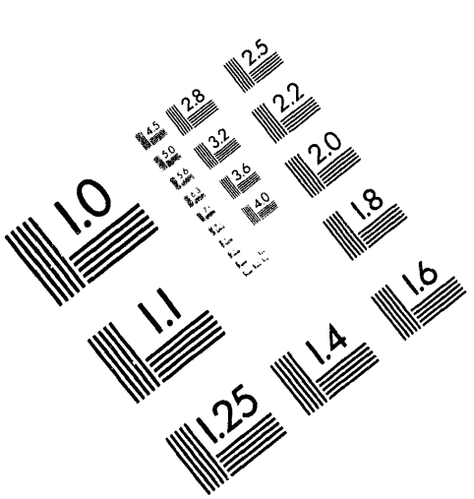

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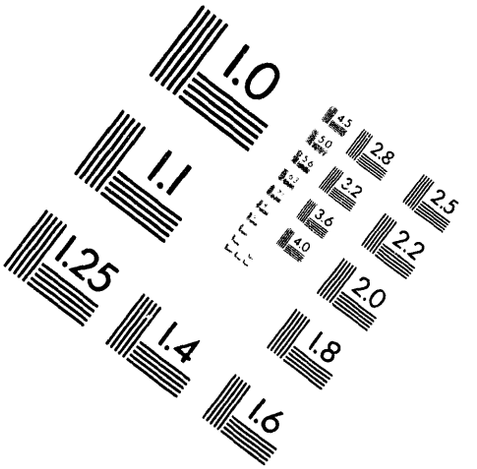

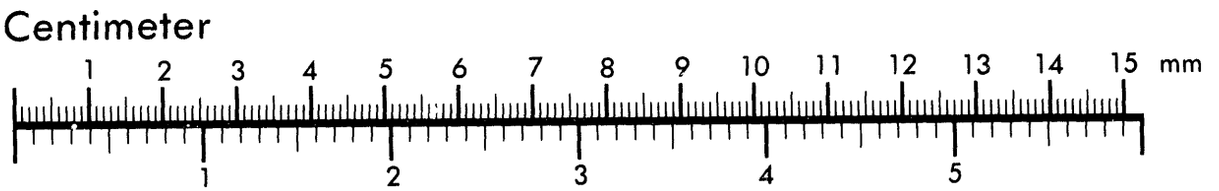

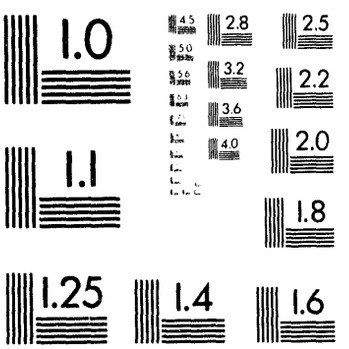

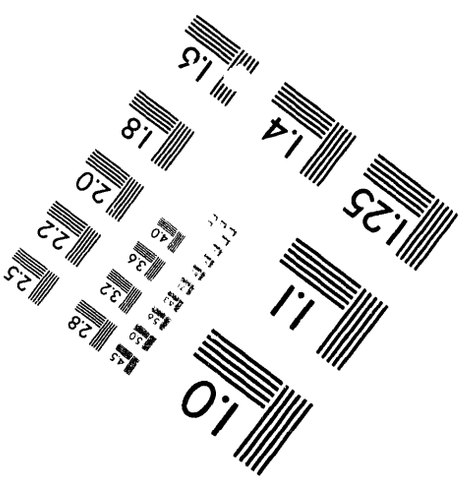

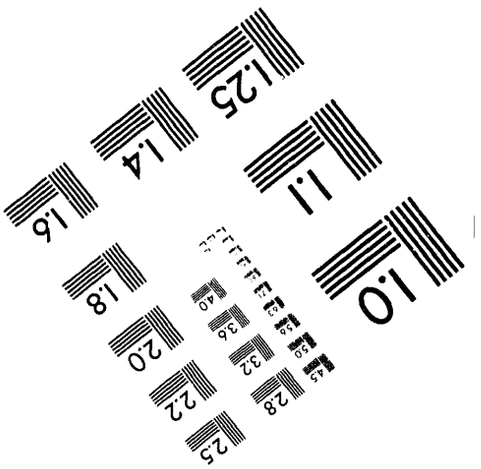

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DOE/ER/13904--T2

TRANSPOSON TAGGING OF DISEASE RESISTANCE GENES
FINAL REPORT
Richard Michelmore. FG03-88ER13904. 5/1/88 TO 4/30/93.

Goals.

To develop a transposon mutagenesis system for lettuce and to clone and characterize disease resistance genes by transposon tagging.

Background and Overview.

At the onset of the project, the best method for isolating genes of unknown function seemed to be by insertional mutagenesis. No transposons had been characterized from lettuce and it had been recently demonstrated that *Ac* from corn could transpose in tobacco. Several transposable elements had been cloned from other plants and animals. Therefore, we concentrated on working with heterologous elements.

Lettuce downy mildew remains one of the best genetically characterized plant-fungal interactions. Thirteen dominant genes for resistance in the host, *Lactuca sativa*, are matched by dominant avirulence genes in the causal fungus, *Bremia lactucae*. Many other sources of resistance are available and in various stages of characterization. The majority of the resistance genes mapped so far are located in one of three genomic clusters. Sequences are duplicated between these regions indicating a common evolutionary origin and functional similarity.

The original proposal described the development of a transposon mutagenesis system for lettuce and its subsequent use to tag disease resistance genes. The development phase involved characterization and manipulation of *Ac* transposition, identification of whole plant selectable markers for the construction of chimeric non-autonomous elements, and the investigation of the stability of resistance genes.

In the early phases of the project, we introduced several heterologous transposons into lettuce. As more data became available, it became clear that only *Ac* was sufficiently well characterized and likely to transpose in heterologous plant species. Therefore, the majority of our studies were conducted with the *Ac/Ds* system. We made and tested several constructs as well as utilized constructions shown by others to be functional in other plant species. We did demonstrate movement of *Ac* and *Ds* in lettuce; however, they transposed at much lower frequencies in lettuce than in other plant species studied to date. Therefore, further manipulation of the system, particularly for flower specific expression of transposase, is required before a routine transposon system is available for lettuce. We analyzed the efficacy of several selectable markers that function in whole plants. These were used as appropriate. Populations of lettuce were generated and screened to test for the stability of resistance genes and we have isolated several spontaneous mutations. We also identified a resistance gene mutant in plants transformed with a *Ds* element and chimeric transposase gene. This is currently being characterized in detail.

Introduction of Heterologous Transposable Elements into Lettuce.

In addition to the experiments with *Ac/Ds* described below, we have introduced three other heterologous transposons into lettuce. Transgenic plants were generated with the *Mu1* element from corn (courtesy of M. Freeeling, UC Berkeley), the *P* element from *Drosophila* (courtesy G. Rubin, UC Berkeley)and the *Tam3* element from *Antirrhinum majus* (courtesy of C. Martin, John Innes Institute, UK). Subsequently, it became apparent that the *Mu1* element was probably not autonomous and that the *P* element was unlikely to transpose in plants; therefore, these transgenics were not studied further. Twenty-four transgenics containing *Tam3* were analyzed by Southern hybridization. In several plants, hybridization patterns were consistent with at least partial excision; however, in no plant were the patterns in all digests and all probes consistent with precise excision of *Tam3*. Analysis of T_2 populations similarly failed to detect clean transposition. In parallel, *Tam3* was shown not to excise precisely in *Antirrhinum* and tobacco (C. Martin, pers. comm.), therefore experiments with *Tam3* in lettuce were discontinued.

Studies with Wildtype *Ac*.

Our first construct, pMAC, involved wildtype *Ac* from *waxy m-7*. Transgenic plants were analyzed by Southern hybridization and PCR for fragments characteristic of *Ac* excision and reintegration. This approach had been successful in tobacco and tomato as transposition of *Ac* was frequent. We regenerated 75 transgenic plants containing pMAC. No clear evidence of transposition was observed. This was in contrast to the results of two neighboring labs using our construct in tomato (Yoder *et al.*, 1988). A total of 80 T_2 plants from six T_1 plants containing *Ac* were also analyzed by PCR and again no evidence of transposition was observed.

As transposition of *Ac* appeared not to occur frequently in lettuce, the next set of experiments involved an excision assay that had been used to assess excision frequency in tobacco (Jones *et al.*, 1989). This assay relied on the restoration of streptomycin resistance following excision of *Ac* from the promoter region of a chimeric streptomycin phosphotransferase gene (SPT). A total of 65 T_1 plants were generated containing the interrupted SPT gene along with 25 positive controls containing the uninterrupted gene. Sixty-two of the resultant T_2 progeny clearly segregated for streptomycin resistance with similar levels of resistance to the uninterrupted positive controls. Resistance was correlated with the presence of the T-DNA; however, Southern analysis provided no evidence of *Ac* excision. These results suggest that *Ac* was efficiently processed from the mRNA in lettuce; similar processing has now been seen in tobacco but at lower levels and with only partial restoration of antibiotic resistance (J. Jones, pers. comm.). Also, this *Ac* contained a modified *Bgl*II site that had subsequently been shown to reduce transposition in tobacco; therefore, studies with these transgenics were discontinued.

Studies with Chimeric Transposons.

To determine whether transposition in lettuce might be stimulated by expressing the transposase gene from a highly expressed heterologous promoter, the next series of experiments attempted to transactivate a *Ds* element that interrupted a chimeric GUS reporter gene using a transposase gene expressed from either a 35S or *Hsp70* promoters.

These constructs had been shown to function well in tomato by M. Lassner in J. Yoder's lab (UC Davis). We introduced each of these constructs into lettuce and crossed T_1 plants expressing transposase with plants containing faithful integrations of the *Ds* element. Only two out of the 48 F_1 plants that were recovered contained both components. Over 90 F_2 plants were analyzed but no evidence of transposition was detected.

Whole Plant Selectable Markers.

We tested the efficacy of several whole plant selectable markers in lettuce for use either within a chimeric *Ds* element or as reporter genes for excision (Yang, 1992). Resistance to the antibiotics, kanamycin, G418, or hygromycin is readily assayed in callus but is less easy to evaluate at the whole plant level. The difference between levels at which all genotypes grow and those at which no genotypes grow is narrow and the optimal level varies from transformant to transformant. A chimeric *Mas*5':SPT gene (Courtesy of J. Jones, Sainsbury Lab., UK) conferred adequate levels of resistance to the antibiotic streptomycin to allow selection of seedlings expressing this gene. A spectinomycin resistance gene expressed from a 35S promoter (also courtesy of J. Jones) provided clear resistance to this antibiotic (Yang *et al.*, 1993). The acetolactate synthase gene from chlorsulfuron-resistant *Arabidopsis thaliana* (courtesy of C. Sommerville, DOE Labs. Michigan State) conferred high levels of resistance to the herbicide over a wide range of concentrations. The *bar* gene from *Streptomyces hygroscopicus* with the *Nos* promoter (courtesy of J. Jones) provided resistance to the herbicide Basta and was used to monitor the presence of some of the *Ds* elements. The *brx* gene from *Klebsiella ozaenae* with either the *Mas* or SSU promoter (courtesy of D. Stalker, Calgene Inc.) gave high levels of resistance to the herbicide Bromoxynil. Therefore, genes for resistance to streptomycin, spectinomycin , chlorsulfuron, basta, and bromoxynil are all useful selectable markers in lettuce.

Dissection of *Ac*.

To study the reasons for the apparent lack of transposition in lettuce and to manipulate *Ac* for efficient tagging strategies, various components of *Ac* were analyzed separately. While on sabbatical for six months at CSIRO, Canberra ('89-90), I made a series of constructs to analyze the *Ac* promoter and the effect of the untranslated leader sequence. The constructs were initially tested in rapid transient assays of tobacco and then transformed into tobacco and lettuce for comparison in stable transgenics.

To analyze the activity of the transposase promoter, a variety of promoter fragments were fused to GUS with and without four repeats of the octopine synthase (OCS) enhancer region. No activity was detected in transient assays in constructs lacking the OCS enhancer. All constructs with the OCS enhancer provided significant levels of GUS activity. The presence of the leader sequence consistently resulted in slightly but not dramatically lower levels of activity in these transient assays.

A second series of constructs were made to investigate the long untranslated leader sequence of *Ac*. Three fragments of leader sequence were introduced into a highly

expressed OCS enhancer:35S promoter:GUS fusion that contained a minimal leader sequence. Surprisingly, all these fragments considerably increased expression. Whether this was a sequence specific effect was not determined. However, these studies did confirm the first series of experiments that the *Ac* leader sequence did not markedly decrease expression in transient assays.

A subset of *Ac* promoter and leader reporter constructs were transformed into tobacco and lettuce to compare their activities when stably integrated into the chromosome. No activity was detected in constructs lacking the OCS enhancer. Medium levels of expression were detected in constructs with the enhancer element. One fragment of the leader sequence increased expression as it had in the transient assays; however, the whole leader fragment considerably decreased GUS activity. This suggests some of the effects of the long untranslated leader are at the transcriptional rather than the translational level. The results in lettuce paralleled those in tobacco except that the GUS activities on a per mg protein basis were lower in lettuce.

Demonstration of *Ac/Ds* Excision in Lettuce Callus.

The next series of experiments to detect transposition in lettuce involved the pKU series of experiments used by B. Baker *et al.* (1987) to demonstrate *Ac* excision in tobacco as well as some derivatives made by J. Ellis (CSIRO, Canberra). These constructs provided positive selection for excision events and therefore the detection of rare excision. Excision of *Ac* or *Ds* restored kanamycin resistance and therefore the ability of callus to grow on this antibiotic. Kanamycin resistant lettuce callus was detected following excision of *Ac* or transactivated *Ds* (Yang *et al.*, 1993b). Transposition was confirmed by Southern analysis, PCR, and sequencing the excision site. These experiments demonstrated that *Ac* was capable of transposition in lettuce and nothing was inherently lacking that prevented transposition. Infrequent transposition may have been due to low promoter activity or inefficient processing.

Demonstration of *Ds* Transactivation in Lettuce Plants.

The final series of experiments to study transposition in lettuce utilized a two element system based on advanced constructs made in the lab of J. Jones (Norwich, UK). The genomic coding region of *Ac* was expressed from the 35S promoter which resulted in elevated levels of transposition (Scofield *et al.*, 1992; Swinburne *et al.*, 1992). *Ds* elements were located in the 5' region of a chimeric spectinomycin resistance gene that had a tobacco RuBP carboxylase small subunit transit peptide to provide cell autonomy for the antibiotic resistance phenotype. The function of these constructs was tested in calli and then numerous plants regenerated carrying each component (Yang *et al.*, 1993a). Crosses were made between 104 independent T_1 plants containing *Ds* and three independent T_1 plants expressing transposase. The excision of *Ds* was monitored using a phenotypic assay of seedlings growing on spectinomycin-containing medium. Green sectors in one-third of the F_1 families indicated transactivation of *Ds* by transposase. Excision was confirmed using PCR and Southern analysis. The lack of green sectors in the majority of F_1 families suggests that many of T-DNA insertion sites are not conducive to transposition. F_1 plants containing both *Ds* and transposase were grown to maturity and

the F_2 seedlings screened on spectinomycin containing medium. Somatic excision was again observed in several progeny but germinal excision was only detected in one F_2 family. Therefore, while *Ds* is capable of transactivation by the transposase in lettuce plants, current constructs result in too infrequent germinal transposition for transposon tagging to be feasible. Further refinement is required before tagging is routine in lettuce. In particular, expression of the transposase from a germline specific promoter would be useful. Such experiments are being conducted by other labs in other species.

Spontaneous Mutation of Resistance Genes.

A problem with experiments to tag resistance genes in other species has been that the spontaneous mutation rate of the target locus has been high, obscuring mutation events due to insertion. Therefore, we generated and screened several populations to investigate the spontaneous mutation rate of *Dm* genes. During the funding period we generated approximately 50,000 F_1 seed that should be heterozygous for five resistance genes. These are currently being screened. We have so far isolated three spontaneous mutations that are now being characterized. Subsequent to the funding period, we generated 2,200 S_2 families of a cultivar containing four *Dm* genes. These families were screened for mutations and four spontaneous mutations identified. Two of these are at *Dm3*. One *Dm3* mutant lacks flanking markers and therefore is a spontaneous deletion mutant; the other retains all the flanking markers presently available and therefore contains no detectable deletion.

Screening of Transformed Families for *Dm* Mutants.

In experiments subsequent to the funding period, we included all the transgenic lines that had been generated as part of this project, in a screen for mutations of *Dm* genes (although we had little evidence for germinal excision of *Ac* or *Ds*). Over 200 T_2 families containing either *Ac* or *Ds* were screened with a mixture of four isolates of *B. lactucae*, each capable of detecting a mutation in a different resistance gene. One family clearly segregated for susceptibility and was shown to lack *Dm3* activity. Marker analysis proved that this was a mutant rather than a contaminant. Genetic analysis demonstrated that the T-DNA carrying a chimeric transposase segregated independently of the mutation but that the T-DNA that had contained a *Ds* element cosegregated with the mutated *Dm3* locus. Recognizing that the probability of inactivating a *Dm* gene by T-DNA insertion is effectively zero, we attempted to demonstrate that the T-DNA was not at the *Dm3* locus and failed. The genomic region flanking the T-DNA was isolated by inverse-PCR and shown to be missing in several deletion mutants at *Dm3* (produced from irradiation with fast neutrons, Okubara *et al.*, 1994). However, the T-DNA mutant lacks no marker flanking *Dm3*. Therefore, this mutant does not contain a detectable deletion and the T-DNA is within a few hundred Kb of *Dm3* or closer. This mutant is currently being characterized in detail.

Conclusions.

Ac/Ds capable of movement in lettuce but further refinement is required before transposon tagging is a routine tool for gene isolation in lettuce. Currently, map-based

cloning seems the more feasible strategy for cloning resistance genes from lettuce and we are placing most of our emphasis on this.

Ac/Ds transposases at a much lower frequency in lettuce than in several other dicotyledenous plants. Two factors probably contributed to the abnormally low rate of transposition in lettuce. One was a position effect of the genomic position of the *Ds* (or *Ac*) element. The other was the level of expression of the transposase gene. This may be a problem for expression of all transgenes in lettuce and warrants further investigation.

We have over 200 independently-generated transgenic lettuce stocks containing either *Ac* or *Ds* stored in the seed vault. These will be valuable resources for transposon mutagenesis using linked elements when chimeric transposase genes providing the necessary timing and level of expression become available.

Two antibiotic resistance genes (streptomycin and spectinomycin resistance) and three herbicide resistance genes, chlorsulfuron (bialophos and bromoxynil resistance) allow efficient selection at the seedling level. Spectinomycin resistance provided a clear indicator for excision of *Ds*.

Adequate numbers of seedlings can be screened for mutagenesis experiments. Several spontaneous (and irradiation-induced) mutants that we have already isolated are proving useful for our continuing efforts to clone resistance genes using a map-based approach. At least one of the mutants has lost flanking genetic markers and is therefore a spontaneous deletion mutant. These mutants will also be fundamental to our studies on mechanisms generating variation within clusters of resistance genes.

One mutant detected in our screen of transgenic lines contains a T-DNA element within a few 100 Kb or closer to a resistance gene. This is currently being characterized in detail.

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